CHAPTER 13

Mammalian RNA Interference

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When viruses infect eukaryotic cells or when transposons and transgenes randomly integrate into host genomes, double-stranded RNA (dsRNA) is frequently produced from the invading genes, either during viral replication or by aberrant transcription from promoters located near the transgene insertion site. Eukaryotes such as plants, protists, and filamentous fungi and invertebrate and vertebrate animals have evolved a cellular defense system that responds to dsRNA and protects their genomes against these invading foreign elements. The dsRNA is rapidly processed by a cellular enzyme to small dsRNA fragments of distinct size and structure (Bernstein et al. 2001), which then direct the sequence-specific degradation of the single-stranded mRNAs of the invading genes (Elbashir et al. 2001a). These short RNA duplexes were therefore named short interfering RNAs (siRNAs). The entire process of posttranscriptional dsRNA-dependent gene silencing is commonly referred to as RNA interference or RNAi (for recent reviews, see Hammond et al. 2001a; Matzke et al. 2001a; Sharp 2001; Tuschl 2001; Waterhouse et al. 2001; Hutvágner and Zamore 2002). In some instances, posttranscriptional gene silencing is also linked to transcriptional silencing (for reviews, see Wassenegger 2000; Bender 2001; Matzke et al. 2001b; Pal-Bhadra et al. 2002).

Experimental introduction of dsRNA into cells has been used to disrupt the activity of cellular genes homologous in sequence to the introduced dsRNA (Fire et al. 1998). RNAi-based reverse genetic analysis now provides a rapid link between sequence data and biological function. RNAi is particularly useful for the analysis of gene function in *Caenorhabditis elegans* (for reviews, see Hope 2001; Kim 2001), but it is also widely used in other invertebrate animals (Kennerdell and Carthew 1998; Ngo et al. 1998; Brown et al. 1999). dsRNA of several hundred base pairs in length is typically required for effective gene silencing (Parrish et al. 2000; Elbashir et al. 2001b). Its application in vertebrate animals, including mammals, has proven to be more difficult because of the presence of additional dsRNA-triggered pathways that mediate nonspecific suppression of gene expression (Caplen et al. 2000; Nakano et al. 2000; Oates et al. 2000; Zhao et al. 2001). Fortunately, these nonspecific responses to dsRNA in vertebrates are not triggered by the siRNAs (Bitko and Barik 2001; Caplen et al. 2001; Elbashir et al. 2001c; Zhou et al. 2002). siRNAs can target genes as effectively as long dsRNAs (Elbashir et al. 2001b) and are widely used today for assessing gene function in cultured mammalian cells or early developing vertebrate embryos (Harborth et al. 2001; Elbashir et al. 2002; Zhou et al. 2002). siRNAs are also promising reagents for developing gene-specific therapeutics (Tuschl and Borkhardt 2002). This chapter concentrates on RNAi as it relates to mammalian systems and on the application of siRNAs for targeting genes expressed in somatic mammalian cell lines.

CONCEPTS AND STRATEGIES

General Mechanism of RNA Interference

Biochemical studies are beginning to unravel the mechanistic details of RNAi. The first cell-free systems were developed using *Drosophila melanogaster* cell or embryo extracts (Tuschl et al. 1999; Hammond et al. 2000; Zamore et al. 2000) and were followed by the development of in vitro systems from *C. elegans* embryos (Ketting et al. 2001) and mouse embryonal carcinoma (EC) cell lines F9 and P19 (Billy et al. 2001). However, the latter two systems do not recapitulate all aspects of RNAi when compared to the *D. melanogaster* systems. Figure 13.1 summarizes the conserved features of the mechanism of RNAi.

Long dsRNA is first processed by Dicer RNase III to siRNAs

Long dsRNAs are first processed to siRNAs by the ribonuclease III (RNase III)-like enzyme Dicer (Hammond et al. 2000; Billy et al. 2001; Ketting et al. 2001). Dicer has an amino-terminal DExH/DEAH RNA helicase domain, a PAZ (Piwi-Argo-Zwille/Pinhead) domain (Cerutti et al. 2000), a tandem repeat of RNase III catalytic domain sequences, and a carboxy-terminal dsRNA-binding motif. In *D. melanogaster* embryo extracts and in Dicer immunoprecipitates of *D. melanogaster* cells, the rate of siRNA formation is ATP-dependent, and siRNAs produced in the embryo lysate in the absence of ATP are one nucleotide longer than in the presence of ATP (Zamore et al. 2000; Bernstein et al. 2001).

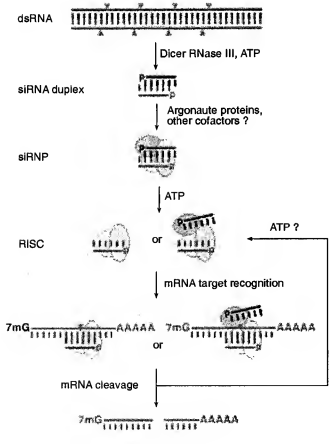

FIGURE 13.1. Model for RNA interference. dsRNA is processed to 21–23-nucleotide siRNA duplexes by Dicer RNase III and possibly other dsRNA-binding factors in an ATP-dependent manner. The siRNA duplexes are incorporated into a siRNA-ribonucleoprotein complex (siRNP) which rearranges, presumably by assistance of a member of the Argonaute protein family and other cofactors such as the catalytic subunit, to the RISC (RNA-induced silencing complex). Subsequently, the mRNA-targeting RISC is formed in an ATP-dependent fashion, which presumably reflects siRNA duplex unwinding. This step could be envisoned to occur in two forms, either by removing one of the strands of the duplex from RISC or by keeping the two siRNA strands spatially separated. After target RNA cleavage, the mRNA cleavage products are released and RISC may be reactivated for another round of catalytic target RNA cleavage.

TABLE 13.1. Size distribution of siRNAs in various eukaryotes

Organism	Predominant length of siRNA (nucleotides)	References
Plants	21–23	Hamilton and Baulcombe (1999); Dalmay et al. (2000a); Hutvágner et al. (2000)
Neurospora crassa	25[a]	Catalanotto et al. (2002)
Drosophila melanogaster	21–22	Elbashir et al. (2001a)
Caenorhabditis elegans	23	Parrish et al. (2000); Ketting et al. (2001)
Trypanosoma brucei	24–26	Djikeng et al. (2001)
Mus musculus	21–22	Yang et al. (2000); Billy (2001); Paddison et al. (2002a)

[a]It is likely that the siRNA length was overestimated because DNA size markers that migrate faster than RNA size markers were used for analysis.

Cytoplasmic extracts from mouse EC cells also process dsRNA to siRNAs (Billy et al. 2001), but the addition of ATP only modestly stimulates the dsRNA processing reaction. The function of ATP during dsRNA processing and the role of the Dicer ATP-dependent RNA helicase domain remain to be elucidated.

Naturally produced siRNA duplexes have two- to three-nucleotide 3′ overhanging ends and contain 5′ phosphate and free 3′ hydroxyl termini (Zamore et al. 2000; Elbashir et al. 2001a,b). The presence of 5′ phosphate and 3′ hydroxyl termini after dsRNA cleavage is a characteristic of all RNase-III-processing reactions (Conrad and Rauhut 2002). In mouse, Dicer is expressed in all stages of development and in a wide variety of adult mouse organs (Nicholson and Nicholson 2002), consistent with RNAi being an innate cellular defense mechanism. Moreover, Dicer has an important role in the processing of microRNAs (miRNAs) (Grishok et al. 2001; Hutvágner et al. 2001; Knight and Bass 2001), which define a new regulatory RNA gene family (for review, see Ambros 2001; Eddy 2001; Grosshans and Slack 2002; Moss 2002; Pasquinelli 2002). Dicer is localized in the cytoplasm of mouse EC cells, indicating that long dsRNA processing as well as miRNA processing reactions occur in the cytoplasm (Billy et al. 2001). Furthermore, invertebrates and vertebrates possess an additional RNase III enzyme, Drosha, which is involved in ribosomal RNA precursor processing (Filippov et al. 2000; Wu et al. 2000). Drosha does not contain a helicase or PAZ domain, but has instead an SR (serine-arginine-rich) and a proline-rich domain.

The production of siRNAs from in-vivo-expressed dsRNAs of transgenes or from synthetic dsRNA delivered into cells is the hallmark of RNAi. Formation of siRNAs has been documented for plants (Hamilton and Baulcombe 1999; Dalmay et al. 2000a; Hutvágner et al. 2000), filamentous fungi (Catalanotto et al. 2002), *C. elegans* (Parrish et al. 2000; Ketting et al. 2001), the trypanosome *Trypanosoma brucei* (Djikeng et al. 2001), and mouse embryonic stem (ES) cells (Yang et al. 2001) and EC cells (Billy et al. 2001; Yang et al. 2001; Paddison et al. 2002a). The length of siRNAs produced varies between 21 and 28 nucleotides (Table 13.1), presumably reflecting structural differences of the various Dicer orthologs. The distinct size and structure of siRNAs presumably reflect the geometric spacing between the active sites of dsRNA-bound dimers of Dicer during dsRNA processing (Blaszczyk et al. 2001; Zamore 2001b).

Cloning and sequencing of small RNAs isolated from *D. melanogaster* and *T. brucei* indicated that siRNAs are indeed produced from dsRNA of retrotransposal origin (Djikeng et al. 2001; Elbashir et al. 2001a), providing additional evidence that RNAi is important for controlling transposable elements (Jensen et al. 1999a,b; Ketting et al. 1999; Tabara et al. 1999; Wu-Scharf et al. 2000).

siRNAs direct sequence-specific target mRNA cleavage after assembly into an endonuclease RNP complex

Analysis of RNAi in *D. melanogaster* extracts has provided compelling evidence that siRNA duplexes, after being generated by Dicer cleavage of dsRNA, are assembled into a multicomponent nuclease, which guides the sequence-specific recognition of the target mRNA (Hammond et al. 2000; Yang et al. 2000; Zamore et al. 2000; Elbashir et al. 2001a). This complex is referred to as the RNA-induced silencing complex (RISC). siRNAs in *D. melanogaster* are predominantly 21 and 22 nucleotides in size (Elbashir et al. 2001a), and when paired to contain the two-nucleotide 3′ overhanging structure are most effective for formation of RISC (Elbashir et al. 2001b). Mammalian systems produce siRNAs of similar size (Yang et al. 2000; Billy et al. 2001; Paddison et al. 2002a), and siRNAs of 21- and 22-nucleotide size represent the most effective sizes for silencing genes expressed in mammalian cells (Caplen et al. 2001; Elbashir et al. 2001c, 2002).

RISC activity formed after incubation of siRNA duplexes in *D. melanogaster* embryo lysate targets homologous sense as well as antisense single-stranded RNAs (ssRNAs) for degradation (Elbashir et al. 2001a,b). The cleavage sites for both sense and antisense single-stranded target RNAs are located in the middle of the region spanned by the siRNA duplexes. The targets are cleaved precisely ten nucleotides upstream of the target position complementary to the 5′ most nucleotide of the sequence-complementary guide siRNA. Importantly, the 5′ end, and not the 3′ end, of the guide siRNA sets the ruler for target RNA cleavage (Elbashir et al. 2001a,b). Furthermore, the presence of a 5′ phosphate at the target-complementary strand of an siRNA duplex is required for siRNA function, and ATP is used to maintain the 5′ phosphates of the siRNAs (Nykänen et al. 2001). Synthetic siRNA duplexes with free 5′ hydroxyls and two-nucleotide 3′ overhangs are so readily phosphorylated in *D. melanogaster* embryo lysates that the RNAi efficiencies of 5′-phosphorylated and nonphosphorylated siRNAs are not significantly different (Elbashir et al. 2001b). However, under certain circumstances, e.g., using 22-nucleotide siRNA duplexes in *D. melanogaster* injection experiments, 5′-phosphorylated siRNAs may show slightly enhanced properties relative to 5′ hydroxyl siRNAs (Boutla et al. 2001). In gene targeting experiments in human HeLa cells, no differences in gene targeting efficiency were observed when comparing 5′ hydroxyl or 5′-phosphorylated siRNAs (Elbashir et al. 2002). Furthermore, in-vitro-transcribed siRNAs that carry 5′ triphosphates are active in human cell gene-silencing experiments (Donzé and Picard 2002; Paddison et al. 2002b). In a recently developed HeLa cell in vitro system (Martinez et al. 2002), siRNA duplexes are also rapidly 5′-phosphorylated, and the siRNAs target RNA cleavage to exactly the same position as in *D. melanogaster* lysates. Taken together, these results indicate that the mechanism of siRNA-mediated target RNA cleavage is conserved between *D. melanogaster* and mammals.

Unwinding of the siRNA duplex must occur prior to target RNA recognition. The initially formed siRNA duplex-containing ribonucleoprotein complex is referred to as siRNP (Nykänen et al. 2001). Analysis of ATP requirements revealed that the formation of RISC on siRNA duplexes requires ATP in lysates of *D. melanogaster*, but once formed, RISC can mediate robust, sequence-specific cleavage of its target in the absence of ATP (Nykänen et al. 2001). This need for ATP probably reflects the unwinding step and probably other conformational requirements. In addition, extensively purified RISC is active in the absence of exogenous nucleotide cofactors (Hammond et al. 2000). However, it is currently unknown whether both of the unwound strands of the siRNA duplex remain associated with RISC or whether RISC only contains a single-stranded siRNA. On the basis of the observations that in *C. elegans* (1) only antisense siRNAs accumulate over time after exposure to dsRNA directed against endogenous genes (Timmons and Fire 1998) and (2) only

one of the two strands constituting an miRNA precursor hairpin accumulates in a stable miRNP complex (Mourelatos et al. 2002), it may be speculated that the latter is true. The simultaneous detection of sense and antisense siRNAs during RNAi and the symmetric cleavage of sense and antisense single-stranded RNA targets may be due to the symmetry of the siRNA duplexes, which may give rise to approximately equal populations of sense and antisense strand-containing RISCs (Elbashir et al. 2001a,b). Alternatively, this observation may also indicate that most siRNAs within a cell are present in the form of duplexes or siRNPs and only a small fraction in the activated form of the RISC.

The identification of the protein components of RISC, especially the catalytic subunit, is important for understanding the function of RISC. Dicer is probably not part of RISC because RISC and Dicer activity can be separated and RISC is unable to process dsRNA to siRNAs (Hammond et al. 2000, 2001b). Furthermore, when siRNAs are used to knock down Dicer in human cells, it does not affect the ability of unrelated siRNAs to target unrelated genes, but as expected compromises the ability to process longer dsRNA and miRNA-like precursors (Hutvágner et al. 2001; Paddison et al. 2002b).

One component associated with RISC from *D. melanogaster* Schneider 2 (S2) cells was identified as Argonaute2 (Hammond et al. 2001b), a member of a large family of proteins (the Argonaute or PPD family) that are characterized by the presence of a PAZ domain and a carboxy-terminal Piwi domain, both of unknown function (Cerutti et al. 2000; Schwarz and Zamore 2002). The PAZ domain is also present in Dicer, and because Dicer and Argonaute2 interact in S2 cells, PAZ may function as a protein-protein interaction motif (Hammond et al. 2001b). Possibly, the interaction between Dicer and Argonaute2 facilitates siRNA incorporation into RISC. The catalytic subunit of RISC still remains to be identified.

Members of the Argonaute gene family have been genetically identified in various organisms and some have important roles during RNAi, whereas others are important in developmental regulation. *C. elegans* contains 24 representatives of this gene family, one of which has been shown to be required for RNAi only, and *rde-1* mutant worms, although defective for RNAi, show no developmental abnormalities. *rde-1* mutants show normal dsRNA processing to siRNAs in vitro (Ketting et al. 2001) as well as in vivo when assayed 12 hours after injection of dsRNA into the syncytial germ line of adult worms (Parrish and Fire 2001). However, in *rde-1* mutant worms exposed to dsRNA by feeding them dsRNA-expressing bacteria, siRNA accumulation was not observed (Tijsterman et al. 2002). However, *N. crassa* RNAi-defective *qde-2* mutants still accumulate siRNAs (Catalanotto et al. 2002). These observations suggest a role for these proteins downstream from dsRNA processing, possibly in stabilization of siRNAs, RISC formation, and/or mRNA targeting. In *Arabidopsis*, Argonaute1 is also involved in posttranscriptional gene silencing (PTGS) and development (Bohmert et al. 1998; Fagard et al. 2000).

In *D. melanogaster*, the Argonaute family has five members and the mRNAs coding for all the Argonaute proteins are maternally deposited (Williams and Rubin 2002). During embryonic development, Argonaute1 and Argonaute2 expression is strong and fairly ubiquitous, whereas Argonaute3, Piwi, and Aubergine zygotic transcription becomes restricted to the presumptive gonad (Williams and Rubin 2002). Argonaute1 mutant flies show defects in early embryo development (Kataoka et al. 2001) and are reduced in their ability to degrade mRNAs in response to dsRNA, although formation of siRNAs was unaffected (Williams and Rubin 2002). Thus, the function of Argonaute1 may be similar to that of Argonaute2, which is associated with RISC (Hammond et al. 2001b). Piwi is required for siRNA formation during silencing of multiple transgenic copies of the *Adh* gene and has a role in some form of transcriptional silencing (Pal-Bhadra et al. 2002). Piwi is furthermore required during *D. melanogaster* development for regulating germ-line stem cell division (Cox et al. 2000). Aubergine is required for the silencing of testis-expressed

Stellate genes by paralogous Su(Ste) tandem repeats involving an RNAi-like mechanism (Aravin et al. 2001) and translational suppression during oogenesis and embryogenesis (Wilson et al. 1996; Harris and Macdonald 2001). Argonaute3 was identified through genome sequencing and remains to be characterized (Williams and Rubin 2002). Two members of the rich Argonaute family in *C. elegans*, *alg-1* and *alg-2*, are required for maturation and stability of miRNAs, which are important regulator molecules that control development (Grishok et al. 2001). The function of most of the other members of this gene family in *C. elegans* (Grishok et al. 2001) remains to be characterized.

The mammalian members of the Argonaute family are also poorly characterized. A rabbit protein from this gene family, eIF2C (Zou et al. 1998), has been implicated in translation initiation. eIF2C was isolated as a major component of a cytoplasmic protein fraction that stimulates the formation of a ternary complex between Met-tRNA, GTP, and the eukaryotic peptide chain initiation factor 2 (eIF2) (Roy et al. 1988; Zou et al. 1998). The human ortholog, eIF2C2, was recently shown to be complexed with Gemin3 (a DEAD-box putative RNA helicase), Gemin4, and mature miRNAs (Mourelatos et al. 2002). The function of this 15S ribonucleoprotein complex (miRNP) is unknown. On the basis of the role of *alg-1/alg-2* in miRNA maturation and stability in *C. elegans* (Grishok et al. 2001), and the presence of a putative RNA helicase in the 15S complex, these miRNPs are involved either in processing miRNAs from longer precursor RNAs and/or in downstream events such as target RNA recognition (Mourelatos et al. 2002). Another member of this family, the human paralog eIF2C1, has been cloned and genetically characterized (Koesters et al. 1999). eIF2C1 is ubiquitously expressed but its function is unknown. Two other members of the mammalian Argonaute family were defined as Miwi (mouse homolog of Piwi), and its human ortholog Hiwi, as well as mouse Mili (Kuramochi-Miyagawa et al. 2001; Sharma et al. 2001). Miwi and Mili were both found in germ cells of adult testis, suggesting that these proteins may function in spermatogenesis (Kuramochi-Miyagawa et al. 2001). Hiwi, which is also expressed in adult testis, was also found expressed in human CD34(+) hematopoietic progenitor cells but not in more differentiated cell populations, again suggesting a role in development of progenitor cells (Sharma et al. 2001). The molecular function and interacting partners of these proteins are currently unknown.

Differences between mammalian RNAi and *C. elegans* or plant RNAi

Plants and worms show systemic silencing, indicating the spread of an amplifiable sequence-specific signal throughout the organisms. The molecular nature of this signal remains to be identified. The signal is most likely RNA in the form of dsRNA or antisense RNA directing new sequence-specific dsRNA synthesis. In *C. elegans*, a putative transmembrane protein, SID-1, was shown to be important for systemic RNAi (Winston et al. 2002). The *sid-1* gene is required to spread gene-silencing information between tissues but not to initiate or maintain an RNAi response. It is possible that SID-1 is involved in endocytosis of the systemic RNAi signal, perhaps functioning as a receptor or as a channel. Consistent with the apparent lack of systemic RNAi in *D. melanogaster* (Kennerdell and Carthew 2000), *sid-1* homologs are absent from the fly genome. The strong similarity to predicted human and mouse proteins, however, suggests the possibility that RNAi could have a systemic component in mammals (Winston et al. 2002).

Screens for genes required for gene silencing in plants, fungi, and worms have identified a family of proteins whose sequences suggest they are RNA-dependent RNA polymerases (RdRPs) (Cogoni and Macino 1999; Dalmay et al. 2000b; Mourrain et al. 2000; Sijen et al. 2001) (see Chapter 9). The discovery of RdRPs in RNAi and PTGS provides a possible explanation for the remarkable efficacy of dsRNA in gene silencing in these

organisms. New dsRNA could be synthesized by RdRPs and thus amplify the silencing process. In *D. melanogaster* and mammals, RdRP genes have not been identified by database analysis.

In *C. elegans*, systemic silencing and signal amplification may also cause transitive RNAi, which is a spreading of silencing outside of the locus targeted by an initiator dsRNA or dsRNA-expression construct (Sijen et al. 2001) (see also Chapter 9). Transitive RNAi is accompanied by the formation of secondary siRNAs, which derive from newly synthesized dsRNA presumably due to RdRP activity. Although this appears to have important implications for RNAi-based analysis of gene function, because silencing may spread between genes that share homologous sequences, phenotypic analysis of a large set of silenced genes in *C. elegans* suggests that transitive RNAi between naturally occurring homologous gene sequences is probably of no major concern (Fraser et al. 2000; Gönczy et al. 2000). It was also suggested that siRNAs might prime novel dsRNA synthesis (Lipardi et al. 2001; Sijen et al. 2001). However, it should be pointed out that siRNAs, in comparison to longer dsRNAs, are extremely poor initiators of gene silencing in *C. elegans* (Parrish et al. 2000; Tijsterman et al. 2002).

Biochemical evidence for RdRP acivity in *D. melanogaster* was recently reported (Lipardi et al. 2001), although classical RdRP genes presumably encoding such activity appear to be lacking from the *D. melanogaster* genome. Despite the postulated target-RNA-dependent dsRNA synthesis, which could potentially lead to amplification of the silencing signal (Lipardi et al. 2001), biochemical evidence for spreading of silencing outside of regions targeted by dsRNAs has not been observed in similar biochemical systems (Zamore et al. 2000; Elbashir et al. 2001a,b; Zamore 2001a). Our attempts to detect polymerization products upon incubation of internally radiolabeled siRNAs with target RNA and nucleoside triphosphates in *D. melanogaster* embryo lysate or HeLa cell lysate, under conditions where dsRNA or siRNAs mediated target RNA degradation, were never successful (Martinez et al. 2002). Additional evidence against propagation of gene silencing in mammalian cells is the ability of siRNAs to specifically silence various isoforms expressed at the same time in the same cell (Kisielow et al. 2002; J. Harborth, unpubl.). This suggests that gene silencing in *D. melanogaster* and mammals is due to siRNA-mediated degradation of target mRNA by RISC, which itself may well catalyze multiple turnovers.

In some instances, PTGS is also linked to transcriptional gene silencing (TGS) (for reviews, see Wassenegger 2000; Bender 2001; Matzke et al. 2001b; Pal-Bhadra et al. 2002). In plants, TGS causes chromatin modifications of the silenced locus, e.g., increased DNA methylation, and it requires promoter sequences to be targeted by PTGS. RNA-directed DNA methylation is most beautifully demonstrated in an experiment where transgene copies of a viral gene present in the nucleus only become methylated upon infection of the plant by the homologous virus, which has a dsRNA genome and which does not enter the nucleus (Jones et al. 1999; Pelissier and Wassenegger 2000). Mutations in *Arabidopsis* selected for reduced DNA methylation, *ddm1*, an SWI2/SNF chromatin component, and *met1*, the major DNA methyltransferase, relieve TGS (Jeddeloh et al. 1998; Mittelsten-Scheid et al. 1998), and in some cases, also include a stochastic reversal of PTGS (Morel et al. 2000).

Additional links between PTGS and TGS were observed. Transgene arrays in the *C. elegans* germ line are desilenced (Tabara et al. 1999) and appear less condensed in mutant backgrounds for some genes required for RNAi (Dernburg et al. 2000). A similar situation was encountered in *D. melanogaster*, where mutations of the *piwi* gene affected both posttranscriptional as well as transcriptional modes of gene silencing (Pal-Bhadra et al. 2002). Such cases of transgene-induced gene silencing appear to require Polycomb-Group proteins, which are complexes known to be involved in the maintenance of

repressive chromatin structure (Pal-Bhadra et al. 1997; Kelly and Fire 1998). Whether mammalian RNA silencing systems also trigger methylation and chromatin changes remains to be resolved.

Analysis of Gene Function in Mammalian Cells Using RNAi

Mammalian gene function has been determined traditionally by methods such as disruption of murine genes, the introduction of transgenes, the molecular characterization of human hereditary diseases, and targeting of genes by antisense or ribozyme techniques. In addition, microinjection of specific antibodies into cultured cells or binding of antibodies to cell-surface-exposed receptors may provide information on the function of the targeted protein.

It has been difficult to detect potent and specific RNAi in commonly used mammalian cell culture systems applying long dsRNA varying in size between 38 and 1662 bp (Caplen et al. 2000; Ui-Tei et al. 2000; Yang et al. 2001; Paddison et al. 2002a). On the one hand, the apparent lack of RNAi in mammalian cell culture was unexpected, because RNAi exists in mouse oocytes and early embryos (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000), and RNAi-related transgene-mediated cosuppression was also observed in cultured Rat-1 fibroblasts (Bahramian and Zarbl 1999). But, on the other hand, it is known that dsRNA in the cytoplasm of mammalian cells can trigger profound physiological reactions that lead to the induction of interferon synthesis (Lengyel 1987; Stark et al. 1998; Barber 2001). In the interferon response, dsRNA greater than 30 bp binds and activates the protein kinase PKR and 2′,5′-oligoadenylate synthetase (2′,5′-AS) (Minks et al. 1979; Manche et al. 1992). Activated PKR stalls translation by phosphorylation of the translation initiation factors eIF2α, and activated 2′,5′-AS causes mRNA degradation by 2′,5′-oligoadenylate-activated RNase L. These responses are intrinsically sequence-nonspecific with respect to the inducing dsRNA.

In an attempt to bypass these sequence-nonspecific effects, three major strategies were employed. In the first case, cell lines were identified that preserved the characteristics of early embryonic stages and have not yet established their interferon system. In the second case, siRNAs were used that are short enough or have a specific structure to escape detection of the interferon system and do not activate PKR or 2′,5′-AS. In the third case, short miRNA-like stem-loop structures were used, which require processing by Dicer, but were short enough to go undetected by the interferon system.

RNAi in embryonic stem cells and embryonic carcinoma cells

Several hundred base-pair-long dsRNAs, transfected or electroporated into undifferentiated mouse ES cells or mouse EC cell lines F9 and P19, induce specific gene silencing without any apparent sequence-nonspecific side-effects (Billy et al. 2001; Yang et al. 2001; Paddison et al. 2002a). Silencing by introduced dsRNA is generally transient, and mouse ES cells recover from the specific knockdown about 5 days after transfection of the dsRNA, presumably due to dilution of the dsRNA during cycles of cell division (Yang et al. 2001). Although most of the reported experiments were focused on suppression of green fluorescent protein (GFP) reporter genes, one of these studies also demonstrated specific silencing of two of the endogenous subunits of cell surface receptor proteins, integrins α3 and β1 in F9 cells (Billy et al. 2001). These proteins turn over rapidly, and their absence at the cell surface was monitored by simple adhesion assays; the reduction of integrin mRNA or protein levels varied between 60% and 90%.

Long dsRNA can also be expressed from transfected plasmid DNA encoding an inverted repeat of a segment of the targeted mRNA. Two different dsRNA expression strategies

were applied. In one study, the inverted repeat was under the control of a T7 promoter. The linearized dsRNA-encoding plasmid was cotransfected into ES cells together with a plasmid encoding T7 RNA polymerase (Yang et al. 2001). In the other case, hairpin dsRNA synthesis was driven by the strong cytomegalovirus (CMV) polymerase II promoter (Paddison et al. 2002a). Stably transformed cells carrying the G418-selectable dsRNA expression construct were expanded into clonal cell lines, some of which were able to specifically silence the transfected homologous reporter gene.

Together, these examples illustrate the ability to transiently or stably silence genes expressed in ES or EC cells, which may be useful to study aspects of cell biology or cell differentiation in undifferentiated cells.

Analysis of gene function in somatic mammalian cells using siRNAs

As an alternative to reverse genetic approaches with long dsRNAs, siRNAs can be used that are also extremely potent elicitors of gene silencing (Caplen et al. 2001; Elbashir et al. 2001c). In contrast to long dsRNAs, siRNAs do not activate the cellular enzymes PKR and 2′,5′-AS of the interferon system established in most transformed somatic mammalian laboratory cell lines. Standard tissue culture cell lines provide starting points for mammalian functional screens because siRNAs can be effectively delivered by classical gene transfer methodologies such as electroporation or cationic liposome-mediated transfection. Transfection efficiencies greater than 90% are commonly achieved in standard laboratory cell lines provided transfection reagents are used that were specially designed for siRNA or antisense oligonucleotide applications (Elbashir et al. 2002). For small-scale applications, microinjection of siRNAs may represent an alternative. Technical problems due to low transfection efficiencies may also be partially overcome by including cell-sorting protocols after cotransfecting siRNAs together with sorting markers such as GFP expression plasmids. Alternatively, siRNAs targeting cell surface marker proteins may be cotransfected, and loss of the cotargeted cell surface marker may be used to gate knockdown cell populations by cell sorting.

The design of siRNA duplexes that interfere with the expression of a specific gene requires accurate knowledge of at least a 20-nucleotide segment of its encoded mRNA (Figure 13.2) (Elbashir et al. 2001b). Intronic sequences contained in pre-mRNAs are best neglected for targeting, because incompletely spliced mRNAs are normally retained in the nucleus and RNAi is believed to occur predominantly, if not exclusively, in the cytoplasm (Montgomery et al. 1998). Also in mammalian cells, mRNA isoforms can be individually silenced, providing further evidence that siRNA-mediated mRNA degradation is a cytoplasmic event (Kisielow et al. 2002). Sequence information about mature mRNAs may be extracted from expressed sequence tag (EST) databases or can be predicted from genomic sequences using gene prediction programs. However, sequencing errors in single-pass EST sequence data or gene predictions should be kept in mind.

siRNA duplexes composed of 21-nucleotide sense and 21-nucleotide antisense strands, paired in a manner to have a two-nucleotide 3′ overhang, are the most efficient triggers of sequence-specific mRNA degradation in tissue culture systems (Elbashir et al. 2002). The target RNA cleavage reaction guided by siRNAs is highly sequence-specific (Elbashir et al. 2001b). However, not all positions of an siRNA contribute equally to target recognition. Mismatches in the center of the siRNA duplex are most critical and essentially abolish target RNA cleavage (Elbashir et al. 2001c; Brummelkamp et al. 2002; Holen et al. 2002). It should be noted that the effect of the mismatches on the specificity of target RNA cleavage is dependent not only on the position of the mismatch relative to the target RNA cleavage site, but probably also on steric or thermodynamic effects that are dependent on the nature of the mismatch. In contrast to mismatches in the paired

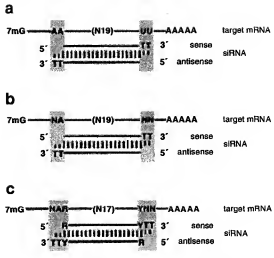

FIGURE 13.2. Selection of siRNA duplexes for mRNA targeting. (*a*) Design of siRNA duplexes for target mRNAs that contain the sequence AA(N19)UU. (*b*) Design of siRNA duplexes in the absence of AA(N19)UU target sequences. As long as one adenosine is present in the targeted region, siRNA duplexes with 3′-TT overhangs can be used without effect on the specificity of target recognition or RNAi efficiency. (*c*) Design of siRNA duplexes that could later be expressed by cloning the sequence into H1 or U6 polymerase III expression cassettes. R and Y indicate purine and pyrimidine nucleotides, respectively; N represents any of the four ribonucleotides.

region of siRNAs, the 3′ nucleotide of the siRNA strand (position 21) that is complementary to the single-stranded target RNA does not contribute to the specificity of target recognition (Elbashir et al. 2001b; Holen et al. 2002). As may be expected, the sequence of the unpaired two-nucleotide 3′ overhang of the siRNA strand with the same polarity as the target RNA is not critical for target RNA cleavage because only the antisense siRNA strand guides target recognition (Elbashir et al. 2001b; Holen et al. 2002). Thus, only the penultimate position of the antisense siRNA (position 20) needs to match the targeted sense mRNA.

Selection of the targeted region is currently a trial-and-error process, but with a likelihood of 80–90% success given a large enough random selection of target genes (Harborth et al. 2001). In every single case, however, the half-life of the targeted gene product, its abundance, or the regulation of its expression must be considered. For example, in an attempt to knock down the strongly expressed and stable intermediate filament protein vimentin, only two out of four randomly selected siRNAs were effective (Harborth et al. 2001). Similar difficulties in finding amenable target sites within the human coagulation trigger tissue factor (TF) mRNA were reported (Holen et al. 2002). Interestingly, there was no apparent correlation between siRNA efficacy and computer-predicted targeted mRNA secondary structure.

Our research group selects target regions such that siRNA sequences may contain uridine residues in the two-nucleotide overhangs (Figure 13.2). Uridine residues in the two-nucleotide 3′ overhang can be replaced by 2′-deoxythymidine without loss of activity, which significantly reduces the cost of RNA synthesis and may also enhance nuclease resistance of siRNA duplexes when applied to mammalian cells (Elbashir et al. 2001c). Another rationale for designing siRNA duplexes with symmetric TT overhangs is to ensure that the sequence-specific endonuclease complex (RISC) is formed with an

TABLE 13.2. Human and animal cell lines in which siRNA triggers silencing

Cell line	Tissue origin	Reference
A-431	human epidermoid carcinoma	Elbashir et al. (2002)
A549	human lung carcinoma	Bitko and Barik (2001)
BV173	human B-precursor leukemia	Tuschl and Borkhardt (2002)
C-33A	human papillomavirus-negative cervical carcinoma	Sui et al. (2002)
CA46	human Burkitt's lymphoma	Tuschl and Borkhardt (2002)
Caco2	human colon epithelial cells	Moskalenko et al. (2002)
CHO	Chinese hamster ovary	Elbashir et al. (2002)
COS-7	African green monkey kidney	Elbashir et al. (2001c)
FS	rat fibroblast	Harborth et al. (2001)
H1299	human nonsmall cell lung carcinoma	Sui et al. (2002)
HaCaT	human keratinocyte cell	Holen et al. (2002)
HEK 293	human embryonic kidney	Elbashir et al. (2001c)
HeLa	human papillomavirus-positive cervical carcinoma	Elbashir et al. (2001c)
Hep3B	human hepatocellular carcinoma	Bakker et al. (2002)
HUVEC	human umbilical vein endothelial cells	Ancellin et al. (2001)
IMR-90	human diploid fibroblast	Paddison et al. (2002b)
K562	human chronic myelogenous leukemia, blast crisis	Tuschl and Borkhardt (2002)
Karpas 299	human T-cell lymphoma	Tuschl and Borkhardt (2002)
MCF-7	human breast cancer	Hirai and Wang (2002)
MDA-MB-468	human breast cancer	Hirai and Wang (2002)
MV-411	human acute monocytic leukemia	Tuschl and Borkhardt (2002)
NIH-3T3	mouse fibroblast	Elbashir et al. (2001c)
P19	mouse embryonic carcinoma	Yu et al. (2002)
SD1	human acute lymphoblastic leukemia	Tuschl and Borkhardt (2002)
SKBR3	human breast cancer	Elbashir et al. (2002)
U2OS	human osteogenic sarcoma cell	Martins et al. (2002)

approximately equal ratio of sense to antisense target RNA-cleaving complexes (Elbashir et al. 2001a,b). This is a precaution, because we do not understand the rules that govern sense versus antisense targeting RISC formation. Other sequences within the two-nucleotide overhangs are also functional and may be preferred if a specific site is targeted, for example, within the mRNA of a fusion gene or a polymorphic or mutated allele.

Analysis of gene function in cultured somatic mammalian cells using siRNAs is now being described in a rapidly growing number of independent studies (see Table 13.2). Cells that show dramatically reduced target protein levels are referred to as knockdown cells, in contrast to knockout cells that are fully deficient for the genetic locus encoding a specific protein. The first broad application of siRNAs for the analysis of cytoskeletal proteins showed that several of these proteins were essential for cell growth (Harborth et al. 2001). But even when nonessential genes were targeted, specific secondary phenotypes were observed in cultured cells that were identical to phenotypes previously observed in mouse gene knockout cells. Furthermore, using siRNAs directed against mitotic proteins, it was possible to reproduce cellular phenotypes that recapitulate the phenotype induced by small-molecule inhibitors specific to the protein encoded by the targeted mRNA (Harborth et al. 2001). These early examples illustrated the value of siRNAs for analysis of mammalian gene function. Subsequently, knockdown of proteins with siRNAs was used for studying

- DNA damage response and cell cycle control (Cortez et al. 2001; Brummelkamp et al. 2002; Mailand et al. 2002; Porter et al. 2002; Stucke et al. 2002; Zou et al. 2002),

- general cell metabolism (Ancellin et al. 2001; Bai et al. 2001),
- signaling (Habas et al. 2001; Li et al. 2001; Martins et al. 2002),
- the cytoskeleton and its rearrangement during mitosis (Du et al. 2001; Harborth et al. 2001),
- membrane trafficking (Short et al. 2001; Moskalenko et al. 2002),
- transcription (Ostendorff et al. 2002), and
- DNA methylation (Bakker et al. 2002).

siRNAs were also used to assess the role of proteins in host-virus interactions (Bitko and Barik 2001; Garrus et al. 2001) or during other disease-causing events such as the expression of polyglutamine in neurodegenerative disorders (Caplen et al. 2002). The breadth and depth of these applications emphasize the key role that siRNAs will have in the functional characterization of gene products and for defining their roles in basic cellular events and disease-related processes in the postgenomic era.

Analysis of gene function in somatic mammalian cells expressing siRNAs or short hairpin RNAs

Until recently, siRNAs for gene targeting experiments have only been introduced into cells via classic gene transfer methods, such as liposome-mediated transfection, electroporation, or microinjection, that require chemical or enzymatic synthesis of siRNAs. Protein knockdowns mediated by exogenous siRNAs are transient because the targeted protein levels of siRNA-treated cells recover, typically between 5 and 7 days after siRNA transfection, i.e., after 7–10 rounds of cell division (Elbashir et al. 2002; Holen et al. 2002; Kisielow et al. 2002). Alternatively, small RNA molecules may also be expressed in the cell. This is possible by cloning the siRNA templates into RNA polymerase III (pol III) transcription units, which are based on the sequences of the natural transcription units of the small nuclear RNA U6 or the human RNase P RNA H1. Two approaches are available for expressing siRNAs: (1) The sense and antisense strands constituting the siRNA duplex are transcribed from individual promoters (Figure 13.3a) (Lee et al. 2002; Miyagishi and Taira 2002; Yu et al. 2002) or (2) siRNAs are expressed as fold-back stem-loop structures that give rise to siRNAs after intracellular processing by Dicer (Figure 13.3b) (Brummelkamp et al. 2002; Paddison et al. 2002b; Paul et al. 2002; Sui et al. 2002; Yu et al. 2002). The endogenous expression of siRNAs from introduced DNA templates overcomes some limitations of exogenous siRNA delivery, in particular the transient loss of phenotype.

U6 and H1 RNA promoters are members of the type III pol III promoters (Medina and Joshi 1999; Paule and White 2000). These promoters are unusual because all promoter elements, with the exception of the first transcribed nucleotide (+1 position), are located upstream of the transcribed region so that almost any inserted sequence shorter than 400 nucleotides can be transcribed. These promoters are therefore ideally suited for expression of siRNAs or approximately 50-nucleotide siRNA stem-loop precursors. The U6 promoter and the H1 promoter are different in size, but they contain the same conserved sequence elements or protein-binding sites (Myslinski et al. 2001). The +1 nucleotide of the U6-like promoters is always guanosine, and always adenosine for H1. Interestingly, changing the +1 adenosine to U, C, or G within H1-expressed stem-loop sequences did not seem to affect gene silencing, suggesting that H1 promoters may be more flexible than U6 promoters for +1 sequence changes or may be able to initiate transcription at the

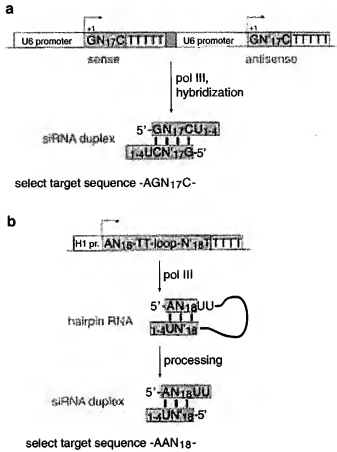

FIGURE 13.3. Endogenous expression of siRNAs. (*a*) Expression cassette for sense and antisense siRNAs using the U6 snRNA promoter (Lee et al. 2002; Miyagishi and Taira 2002). (*White box*) The 250-bp U6 snRNA promoter; (*blue box*) the pol III terminator signal composed of a run of thymidines; (*gray box*) the spacer between the sense and antisense expression element; (*red box*) siRNA elements. The target site preferably selected for optimal vector design is indicated at the bottom. (*b*) H1 RNA-based pol III cassette for expressing hairpin RNAs that are subsequently processed to siRNAs (Brummelkamp et al. 2002). The H1 RNA pol III promoter is only 100 bp in size, but it contains all the essential sequence motifs present in the U6 snRNA promoter (Myslinski et al. 2001). Hairpin RNAs with gene-silencing properties were also obtained by using a U6 promoter (Paddison et al. 2002b; Paul et al. 2002; Sui et al. 2002; Yu et al. 2002). In this case, transcript synthesis was initiated with a +1 guanosine, and the 3′ end of the sense strand was joined by short oligonucleotide loops with the antisense strand.

first downstream purine nucleotide encoded by the template DNA (Brummelkamp et al. 2002). RNA transcription is terminated when pol III encounters a run of four or five thymidines by incorporation of only some of the encoded uridines (Myslinski et al. 2001).

DNA constructs encoding 19-bp stem-loop sequences with 3′-overhanging uridines can silence target genes as effectively as synthetic siRNAs (Brummelkamp et al. 2002;

TABLE 13.3. Strategies employed for endogenous expression of siRNA or short hairpin RNAs

Promoter	Preferred targeting structure[a]	References
H1 pol III	19-bp/9-nucleotide (equivalent to 21-bp/5-nucleotide) stem-loop	Brummelkamp et al. (2002)
U6 pol III	21-nucleotide sense and 21-nucleotide antisense siRNA transcribed by separate promoters	Lee et al. (2002); Miyagishi and Taira (2002)
U6 pol III	4-nucleotide/19-bp stem-loop	Paul et al. (2002)
U6 pol III	29-bp/8-nucleotide stem-loop	Paddison et al. (2002b)
U6 pol III	21-bp/6-nucleotide (or 23-bp/2-nucleotide) stem-loop	Sui et al. (2002)
U6 pol III	19-bp/2-nucleotide stem-loop	Yu et al. (2002)

[a]The position of the loop and the length of the target-sequence-containing stem are indicated. If the length of the loop is given prior to the length of the stem, it indicates that the loop connects the 5′ end of the sense strand to the 3′ end of the antisense (targeting) strand; if the length of the stem is followed by the loop size, the 5′ end of the antisense strand is connected to the 3′ end of the sense strand.

Paul et al. 2002; Yu et al. 2002), but blunt-ended duplexes with up to 29 bp are also able to mediate RNAi in cultured cells (Paddison et al. 2002b). Intracellular processing of plasmid-encoded hairpin RNAs requires Dicer RNase III because Dicer knockdown cells do not support hairpin-mediated target gene silencing (Paddison et al. 2002b). The size, orientation, and sequence of the loop affect the efficiency of gene silencing in many cases. However, the precise processing rules for short hairpin RNAs are not fully understood, so that variations in targeting efficiency may in part be due to variation of the target cleavage site (see Table 13.3). The efficiency of target RNA cleavage, even for single-nucleotide displacements of the siRNA relative to the target, is quite variable (Elbashir et al. 2001b). Furthermore, before precise processing rules for hairpin processing have been elucidated, it should be cautioned that loop spacer elements, which are typically noncognate to the target, as well as noncognate base pairs adjacent to the central paired regions, may affect specificity or impair efficacy of the produced siRNAs.

Using siRNA expression systems, it is possible to extend the periods of persistent suppression or stable loss-of-function phenotype by producing stable cell lines propagating the siRNA expression cassettes. Miyagishi and Taira (2002) suppressed β-catenin, a protein involved in cadherin-mediated cell-cell adhesion, for more than 1 week. The β-catenin-targeting siRNA strands were expressed from a plasmid containing the Epstein-Barr virus (EBV) DNA replication origin, and the plasmid was propagated in cells stably expressing EBV nuclear antigen 1 (EBNA-1). Two other groups produced cells that stably suppressed p53 protein, an important protein involved in the cellular response to ionizing irradiation DNA damage (Brummelkamp et al. 2002; Paddison et al. 2002b). Silencing of p53 was observed for more than 2 months in antibiotic-selected, stably transfected cell clones, also indicating that long-term expression of siRNAs is nontoxic to cells (Brummelkamp et al. 2002).

In summary, stable knockdown cells of nonessential proteins are of great value for studying inducible processes such as UV irradiation damage response, host-pathogen interactions, or cell differentiation and will enable synthetic lethality screens in human cells. The establishment of clonal cell lines with inducible siRNA expression systems should add additional value to the siRNA repertoire, because it would be possible to synchronize the knockdown of entire cell populations, and because essential genes may be

targeted. Strategies for the regulated expression of small RNAs have already been described (Meissner et al. 2001; Yarovoi and Pederson 2001; Miyagishi and Taira 2002) and provide a starting point for such developments.

To decide whether a transient or more long-term silencing strategy should be chosen, the following should be considered. Transfection of plasmid DNA relative to synthetic siRNAs may appear advantageous in view of the danger of RNase contamination or the current costs of chemically synthesized siRNAs or siRNA transcription kits. For practical applications, however, the additional time involved in preparing and amplifying siRNA expression vectors and the transfection efficiency of plasmids relative to siRNAs must also be considered. Furthermore, targeting of essential genes causes arrest in cell growth or cell death within 1–3 days after delivery of siRNAs, thus making long-term silencing unnecessary.

Considering all the pros and cons of expressed versus synthetic siRNAs, it is probably most effective to initiate the search for highly effective siRNAs with synthetic, ready-to-use duplex RNAs of defined sequence and length, and select the synthetic sequences such that they are already compatible with the sequence requirements for expression within U6 or H1 RNA expression cassettes. Such constraints represent (1) the +1 position of U6 snRNA for a guanosine (Paule and White 2000; Paddison et al. 2002b) and probably the +1 position for adenosine in H1 RNA and (2) the 3′-terminal uridines encoded by the oligothymidine pol III terminator signal sequence (Paule and White 2000).

In summary, the possibility for stable expression of siRNAs has paved the way to new gene therapy applications such as treatment of persistent viral infections. Incorporation of siRNA expression cassettes into (retro)viral vectors may allow targeting of primary cells previously resistant or refractory to siRNA or plasmid DNA transfection. Because of the automation developed for high-throughput sequence analysis of the various genomes, the DNA-based methodology may also provide a cost-effective alternative for automated genome-wide loss-of-function phenotypic analysis, especially when combined with miniaturized array-based phenotypic screens (Ziauddin and Sabatini 2001).

SUMMARY

RNAi represents an evolutionarily conserved cellular defense mechanism for controlling the expression of alien genes in almost all eukaryotes including humans. RNAi is triggered by dsRNA and causes sequence-specific mRNA degradation of single-stranded target RNAs homologous in response to dsRNA. The mediators of mRNA degradation are siRNAs, which are produced from long dsRNA by enzymatic cleavage in the cell. siRNAs are approximately 21 nucleotides in length and have a base-paired structure with two-nucleotide 3′ overhangs. Although they were discovered only recently, siRNAs have already revolutionized functional analysis of mammalian gene function and are rapidly moving toward genome-wide systematic analysis of gene function in cultured cells. siRNAs may soon become a valuable tool for target validation beyond classical tissue culture cell lines. Similar to humanized monoclonal antibody strategies as therapeutic platform technology, siRNAs may provide an interesting solution for gene-specific drug development, especially before the availability of highly specific small-molecule inhibitors.

A selection of protocols, modified from Elbashir et al. (2002), is presented for targeting endogenous genes in mammalian somatic cells.

TECHNIQUES

PROTOCOL 1: SELECTION OF siRNA SEQUENCES

To target a specific mRNA for degradation, a portion of the mRNA target sequence must be known and a segment of the target mRNA must be chosen that will be used for targeting by the cognate siRNA duplex. The siRNA selection process has recently been automated by Bingbing Yuan and Fran Lewitter at the Whitehead Institute (Cambridge, Massachusetts) and a Web Site (http://jura.wi.mit.edu/bioc/siRNA/home.php) has been made publicly available. This software allows the user to define sequence motifs and G/C content, to search siRNAs against the human and mouse genome databases to prevent mistargeting, and to exclude single-nucleotide polymorphic sites.

Procedure

1. Select the target region from the open reading frame of a desired cDNA sequence, preferably 50–100 nucleotides downstream from the start codon.

   It is conceivable that 5′UTRs or 3′UTRs or regions close to the start codon are less effectively targeted by siRNAs, as these may be richer in regulatory protein-binding sites. UTR-binding proteins and/or translation initiation complexes could interfere with binding of RISC to the target RNA.

   If the intent, however, is to rescue a knockdown phenotype by reintroduction of a plasmid coding for a mutant or tagged form of the targeted gene, it may be preferable to target regions in the UTRs. Preparation of rescue constructs by deletion of terminal untranslated sequences is easier than the introduction of silent mutations within the targeted region of a coding segment. In a recent survey of 3′UTR-localized targeting sites of more than 40 essential genes, we found that 3′UTRs are in fact as effectively targeted as coding regions (M. Hossbach, S. Elbashir, T. Tuschl, unpubl.).

2. Search for sequences 5′-AA(N19)UU, where N is any nucleotide, in the mRNA sequence, and ideally choose those with ~50% G/C content (see Figure 13.2a). Nevertheless, 32–79% G/C content has also worked well in our hands. Highly G-rich sequences should be avoided because they tend to form G-quartet structures.

   If there are no 5′-AA(N19)TT motifs present in the target mRNA, search for 5′-AA(N21) or 5′-NA(N21) sequences (Figure 13.2b). Independent of the selection procedure described in Figure 13.2, synthesize the sense siRNA as 5′-(N19)TT, and the sequence of the antisense siRNA as 5′-(N′19)TT, where N′19 denotes the reverse complement sequence of N19. N19 and N′19 indicate ribonucleotides, and T indicates 2′-deoxythymidine.

   If the intent, however, is to also express a chemically synthesized siRNA using pol-III-based expression vectors, select the targeted sequence as 5′-NAR(N17)YNN (Figure 13.2c), where R and Y indicate purine and pyrimidine nucleotides, respectively. The sense siRNA is then

synthesized accordingly as 5′-R(N17)YTT and the sequence of the antisense siRNA as 5′-R′(N′17)Y′TT.

3. Perform a BLAST search (www.ncbi.nlm.nih.gov/BLAST) using the selected siRNA sequences as the input against EST libraries or mRNA sequences of the respective organism to ensure that only a single gene is targeted.
4. (*Although optional, this step is recommended*) Synthesize several siRNA duplexes to control for the specificity of the knockdown experiments.

   Those siRNA duplexes that are effective for silencing should produce exactly the same phenotype. Furthermore, a nonspecific siRNA duplex may be needed as a control. It is possible to reverse the sequence of an effective siRNA duplex or to use a siRNA duplex that targets a gene absent from the selected model organism, e.g., GFP or luciferase. We have used an siRNA duplex targeting firefly luciferase as a control for targeting endogenous genes in mammalian cells because the firefly luciferase gene was not present in the targeted cells (Elbashir et al. 2002c).

Troubleshooting

If the siRNA does not work, first verify that the target sequence and the cell line used are derived from the same organism. According to a recent study, there is a high probability of using the wrong cell line (Masters et al. 2001). In addition, make sure that the mRNA sequence used for selection of the siRNA duplexes is reliable; it could contain sequencing errors, mutations (e.g., in cancer cells), or polymorphisms.

PROTOCOL 2: ANNEALING siRNAS TO PRODUCE siRNA DUPLEXES

Sense and antisense siRNA strands are annealed to form a duplex prior to transfecting them into cultured cells.

Procedure

▼ CAUTION
See Appendix for appropriate handling of materials marked with <!>.

MATERIALS

REAGENTS

2x Annealing buffer
- 200 mM potassium acetate
- 4 mM magnesium acetate <!>
- 60 mM HEPES-KOH (pH 7.4) <!>

Ethidium bromide solution (1% w/v) (aqueous) <!>

NuSieve GTG agarose (BMA, Rockland, Maine; www.bmaproducts.com)

Sense and antisense siRNA in H_2O at a concentration >80 μM

Sucrose gel-loading buffer (Sambrook et al. 2001)

5x TBE buffer
- 450 mM Tris base <!>
- 450 mM boric acid <!>
- 10 mM Na_2EDTA

EQUIPMENT

Gel electrophoresis equipment

UV light source

Water bath preset to 90°C

1. Prepare a 20 μM siRNA duplex solution by combining:

   70 μl of 2x annealing buffer

   sense siRNA to 20 μM final concentration

   antisense siRNA to 20 μM final concentration

   sterile H_2O to a final volume of 140 μl.

2. Incubate the reaction for 1 minute at 90°C, followed by 1 hour at 37°C.

   Store unused siRNA duplex solution frozen at –20°C. The siRNA duplex solution can be frozen and thawed many times and does not require any further heat shock treatments. Always keep RNA solutions on ice as much as possible to reduce the rate of RNA hydrolysis.

3. To assess the completeness of the annealing reaction:

   a. Separately load 1 μl of 20 μM sense and antisense siRNAs and 0.5 μl of 20 μM siRNA duplex onto a 4% NuSieve GTG agarose gel. When loading the samples, it is helpful to first dilute the samples with a few microliters of 0.5x TBE buffer and sucrose-loading buffer.

   b. Run the gel in 0.5x TBE buffer at 80 V for 1 hour.

      NuSieve agarose is a low-melting-temperature agarose, which may melt if electrophoresis is performed with excessive electric current.

   c. Detect the RNA bands under UV light after ethidium bromide staining. Preferably, add the ethidium bromide to the 4% gel/0.5x TBE solution at a concentration of 0.4 mg/liter (4 μl of 1% ethidium bromide solution per 100 ml of gel solution) prior to casting the gel.

PROTOCOL 3: CELL CULTURE AND PREPARATION OF CELLS IN 24-WELL PLATES

Transfection of cultured cells with siRNAs and downstream analysis of the knockdown cells are best performed in multiwell tissue culture plates.

Procedure

▼ CAUTION
See Appendix for appropriate handling of materials marked with <!>.

MATERIALS

REAGENTS

- Dulbecco's modified Eagle medium (DMEM) (41966-029, Life Technologies; www.lifetech.com)
- Fetal bovine serum (FBS) (10500-064, Life Technologies)
- Mammalian cell lines (e.g., HeLa S3, HeLa SS6, COS-7, NIH-3T3, HEK 293, CHO, A431, and SKBR3)
- Penicillin and streptomycin (A2212, BioChrom; www.biochrom.com) <!>
- Trypsin-EDTA solution (25300-054, Life Technologies) <!>

EQUIPMENT

- Cell culture flask (175 ml)
- Cell culture plate (24 well)
- Coverslips
  - Optional, see Step 3.
- Incubator (5% CO_2, humidified)

1. Grow mammalian cell lines in a 5% CO_2 humidified incubator at 37°C in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Passage cells regularly to maintain exponential growth.

   Do not exceed a passage number of 30 after unfreezing the stock culture. The number of passages may affect DNA and siRNA transfection efficiencies. Aliquots of cells with low passage number may be stored frozen and can be thawed as needed.

   For general advice on cell culture, see Spector et al. (1999).

2. At least 24 hours before plasmid/siRNA transfection, trypsinize 90% confluent cells grown in a 175-ml cell culture flask with 10 ml of trypsin-EDTA.

3. Dilute the cell suspension 1:5 with fresh DMEM without antibiotics and transfer 500-µl aliquots into each well of a 24-well plate.

   If immunofluorescence assays are planned, grow cells on coverslips placed at the bottom of the 24-well plates prior to addition of the cell suspension.

4. At least 24 hours after seeding the cells, ensure that a confluency of 50–80% is reached, which corresponds to 3×10^4 to 1×10^5 cells per well, depending on the cell line and its doubling time.

PROTOCOL 4: COTRANSFECTION OF LUCIFERASE REPORTER PLASMIDS WITH siRNA DUPLEXES

Before siRNAs are applied to knock down an endogenous gene, it may be important to establish whether the studied cells are susceptible to RNAi. It may be possible that some cell lines have lost the ability to perform RNAi or that cells derived from certain tissues do not support RNAi.

This protocol describes a reporter assay for RNAi in mammalian cells and is based on a published procedure (Elbashir et al. 2002). The quantities of reagents given below are calculated for the transfection of one well of a 24-well plate.

Procedure

MATERIALS

REAGENTS

Dual-Luciferase Assay (E1960, Promega)

LIPOFECTAMINE 2000 (11668-019, Invitrogen; www.invitrogen.com)

OPTI-MEM 1 medium (31985-047, Life Technologies)

Mammalian cells

Plasmids

pGL2-Control plasmid (E1611, Promega; www.promega.com)
pGL3-Control plasmid (E1741, Promega)
pRL-TK plasmid (E2241, Promega)

siRNA duplexes (see Protocol 2)

- GL2 luciferase siRNAs

  sense siRNA: 5′ CGUACGCGGAAUACUUCGAdTdT
  antisense siRNA: 5′ UCGAAGUAUUCCGCGUACGdTdT

- invGL2 siRNAs (inverted sequence of GL2 siRNA as nonspecific control)
  sense siRNA: 5′ AGCUUCAUAAGGCGCAUGCdTdT
  antisense siRNA: 5′ GCAUGCGCCUUAUGAAGCUdTdT
- GL3 luciferase siRNAs
  sense siRNA: 5′ CUUACGCUGAGUACUUCGAdTdT
  antisense siRNA: 5′ UCGAAGUACUCAGCGUAAGdTdT
- RL luciferase siRNAs
  sense siRNA: 5′ AAACAUGCAGAAAAUGCUGdTdT
  antisense siRNA: 5′ CAGCAUUUUCUGCAUGUUUdTdT

EQUIPMENT

Cell culture plates (24-well)

Incubator (37°C, 5% CO_2, humidified)

1. On the day before transfection, culture cells in 24-well plates by completing Steps 2–4 of Protocol 3.
2. On the day of transfection, mix:

   1.0 µg of pGL2-Control plasmid or 1 µg of pGL3-Control plasmid

   0.1 µg of pRL-TK plasmid

   0.21 µg of siRNA duplex (0.75 µl of 20 µM annealed duplex; see Protocol 2)

   50 µl of OPTI-MEM 1 medium

   Reporter plasmids may be amplified in XL-1 Blue (200249, Stratagene; www.stratagene.com) and purified using the QIAGEN EndoFree maxi plasmid kit (www.qiagen.com).
3. In a separate tube, add 2 µl of LIPOFECTAMINE 2000 to 50 µl of OPTI-MEM 1 medium. Mix the tube gently by inverting; *do not* vortex. Incubate the suspension for 5 minutes at room temperature without movement.
4. Combine the solution from Step 2 with the suspension from Step 3. Mix gently by inverting the tube, and then incubate it for 20–25 minutes at room temperature to allow for formation of liposome complexes. Do not exceed a 30-minute incubation time.
5. Add the liposome complexes to the well of cells (from Step 1) without replacing the growth medium and mix gently for 15 seconds by gently rocking the plate. Incubate the plate for 20–48 hours at 37°C in a 5% CO_2 humidified incubator. If cytotoxic effects are expected from the transfection reagent, change the growth medium 5 hours after transfection.
6. To monitor luciferase activity, lyse the cells and measure luciferase expression using the Dual-Luciferase Assay according to the manufacturer's instructions.

   To estimate the transfection efficiency, it is convenient to cotransfect a GFP-coding plasmid together with 0.21 µg of a siRNA duplex noncognate to GFP (e.g., invGL2) and to count the GFP-expressing cells by fluorescence microscopy. Transfection efficiencies for most cell lines described above range from 70% to 90%.

PROTOCOL 5: TRANSFECTION OF siRNA DUPLEXES

In the absence of reporter plasmids, siRNAs are best delivered with transfection reagents developed for delivery of antisense oligodeoxynucleotides. Such reagents are sometimes less toxic than plasmid delivery reagents and may show higher transfection efficiencies than conventional transfection reagents.

Two transfection reagents have been used predominantly in our research group: OLIGOFECTAMINE from Invitrogen and TransIT-TKO siRNA Transfection Reagent from Mirus. The quantities of reagents given below are calculated for the transfection of one well of a 24-well plate.

Procedure

MATERIALS

REAGENTS

Cells (see Step 1)
OLIGOFECTAMINE (Invitrogen, www.invitrogen.com)
or
TransIT-TKO siRNA Transfection Reagent (Mirus, http://genetransfer.com/)
OPTI-MEM 1 medium (31985-047, Life Technologies)
siRNA duplexes (see Protocol 2)

EQUIPMENT

Incubator (5% CO_2, humidified)

1. The day before transfection, complete Steps 2–4 of Protocol 3, except dilute the cell suspension after trypsination of the stock culture 1:10 rather than 1:5 before transferring to the 24-well plate (see Step 3 of Protocol 3). Use a higher dilution to obtain the recommended confluency of 50% for OLIGOFECTAMINE transfection.
2. Mix 3 µl of the 20 µM siRNA duplex (0.84 µg, 60 pmoles) with 50 µl of OPTI-MEM 1.
3. In a separate tube, add 3 µl of OLIGOFECTAMINE (or 4.0 µl of TransIT-TKO) to 12 µl of OPTI-MEM 1. Mix gently and incubate it for 7–10 minutes at room temperature.
4. Slowly add the siRNA solution (Step 2) to the solution prepared in Step 3 and mix gently by inversion; *do not* vortex. Incubate the tube for 20–25 minutes at room temperature to allow for formation of lipid complexes; the solution will turn turbid. Then add 32 µl of fresh OPTI-MEM 1 medium to obtain a final volume of 100 µl and mix gently by inversion.
5. Add the 100 µl of lipid complexes from Step 4 to the well of cells (from Step 1) without replacing the growth medium and mix gently for 30 seconds by gently rocking the plate. Incubate the plate for 2–3 days at 37°C in a 5% CO_2 humidified incubator.

   TransIT-TKO reagent is more difficult to handle than OLIGOFECTAMINE, because the concentrations required for effective transfection also cause cytotoxic effects. Typical side effects of TransIT-TKO siRNA transfection are formation of extended lamellipodia as well as oval-shaped nuclei that appear ~2 days after transfection. These effects are observed using between 4.0 and 4.5 µl of TransIT-TKO reagent.

PROTOCOL 6: IMMUNOFLUORESCENCE DETECTION OF PROTEIN KNOCKDOWN

The preferred way of detecting a gene knockdown is to use a specific antibody that recognizes the targeted gene product.

Procedure

▼ CAUTION
See Appendix for appropriate handling of materials marked with <!>.

MATERIALS

REAGENTS

- Cells from Protocol 5
- Hoechst 33342 (bisbenzimide; 15091, Serva; www.serva.com) <!>
- Methanol, chilled to –10°C <!>
- Moviol mounting medium (Hoechst, www.hoechst.com)
- Phosphate-buffered saline (PBS) (pH 7.1)
  - 137 mM NaCl
  - 7 mM Na_2HPO_4 <!>
  - 1.5 mM KH_2PO_4 <!>
  - 2.7 mM KCl <!>
- Specific primary and secondary antibodies
  - Dilute the antibodies with PBS buffer containing 0.5 mg/ml BSA (A 9706, Sigma) and 0.02% NaN_3 <!>. The secondary antibody is fluorescently labeled.

EQUIPMENT

- Ceramic rack
- Cell culture plate (24-well) carrying knockdown cells on coverslips (from Protocol 5)
- Coverslips
- Filter paper
- Incubator (37°C)
- Nail polish
  - See Step 10.
- Petri dish (13-cm diameter)
  - See Step 3.
- Slides
- Tweezers (Dumont No. 7)
- Upright light microscope
  - For example, a Zeiss Axiophot with an F Fluar 40x/1.30 oil objective and MetaMorph Imaging Software (Universal Imaging Corporation, West Chester, Pennsylvania).
  - Alternatively, a laser-scanning microscope may be used.

1. Fix and permeabilize the knockdown cells.

   a. Use tweezers to remove the coverslips carrying the knockdown cells (from Protocol 5) from the 24-well plate.

   b. Place the coverslips on a ceramic rack and then incubate them in methanol chilled to −10°C for 6 minutes.

      Methanol fixation is suitable for the detection of many cellular proteins, but the optimal fixation procedure may have to be established experimentally for each individual protein (Celis et al. 1998; Spector et al. 1999). We recommend beginning with methanol fixation, which preserves the ultrastructure of the cell and sufficiently permeabilizes the cells for penetration of the antibody.

2. Wash the methanol-fixed coverslips three times in PBS and touch filter paper to the coverslips to remove excess PBS.

3. Place the coverslips in a wet chamber with the cells side facing up. Prepare a wet chamber by soaking filter paper in H_2O and placing it into a 13-cm-diameter Petri dish. Do not allow the specimens to dry out during this procedure.

4. Add 20 µl of appropriately diluted primary antibody on top of the coverslip without touching the cells. Make sure that the solution is evenly spread out over the entire surface of the coverslip. Transfer the closed wet chamber into a 37°C incubator and incubate for 45–60 minutes.

   Antibodies are diluted with PBS containing 0.5 mg/ml bovine serum albumin and 0.02% sodium azide.

5. Place the coverslips again on the ceramic rack and wash them three times with PBS, each for 5 minutes. Touch filter paper to the coverslips to remove excess PBS, and then transfer the coverslips back into the wet chamber.

6. Add 20 µl of appropriately diluted, fluorescently labeled secondary antibody to each coverslip. Incubate the cells in the closed wet chamber for 45 minutes at 37°C.

7. Repeat Step 5.

8. Detect the cell nuclei by chromatin staining. Add 20 µl of 1 µM Hoechst 33342 solution in PBS on top of the coverslip and incubate for 4 minutes at room temperature.

9. Repeat Step 5.

10. Mount two coverslips per slide by placing the coverslips with the cells side facing downward on a drop of Moviol mounting medium. Place a piece of filter paper on top of the slide and press gently on top of the paper to remove excess mounting medium. Glue coverslips to the slide with nail polish.

11. Examine the immunofluorescence staining and take pictures using an upright light microscope. Use identical exposure times for photographing both the silenced cells and the control-treated cells.

    Alternatively, a laser-scanning microscope may be used.

PROTOCOL 7: DETECTION OF PROTEIN KNOCKDOWN BY WESTERN BLOTTING

Knockdown of proteins is frequently associated with impaired cell growth or altered cell morphology, which can be monitored by phase-contrast microscopy. If no alterations in cell growth or cell morphology are observed, immunofluorescence or western blotting can be performed to analyze the depletion of the target protein.

Procedure

▼ CAUTION
See Appendix for appropriate handling of materials marked with <!>.

MATERIALS

REAGENTS

- Blocking solution (5% milk powder in TBST [pH 7.4])
- Dulbecco's modified Eagle medium (DMEM)
- ECL (enhanced chemiluminescent) detection kit (www.amersham.co.uk)
- Electrotransfer buffer
  - 25 mM Tris <!>
  - 192 mM glycine <!>
  - 0.01% SDS <!>
  - 20% methanol <!>
- 2x Laemmli SDS sample buffer (161-073, Bio-Rad; www.bio-rad.com)
- Phosphate-buffered saline (PBS) (pH 7.1)
  - 137 mM NaCl
  - 7 mM Na_2HPO_4 <!>
  - 1.5 mM KH_2PO_4 <!>
  - 2.7 mM KCl <!>
- Ponceau S stain (Sigma) <!>
- Primary antibody
  - See Step 8. If necessary, dilute the antibody in TBST.
- Secondary antibody
  - Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse or HRP-conjugated swine anti-rabbit antibodies (Dako Diagnostika, Hamburg, Germany; www.dako.com)
- siRNA-treated cells cultivated in 24-well plates (from Protocol 5)
- TBST (pH 7.4)
  - 0.2% Tween-20
  - 20 mM Tris-HCl <!>
  - 150 mM NaCl
- Trypsin-EDTA solution (25300-054, Life Technologies) <!>

EQUIPMENT

- Centrifugation tube (1.5 ml)
- Centrifuge
- Electrotransfer equipment
- Enhanced chemiluminescent detection equipment
- Nitrocellulose membrane (Protran BA85 0.45 mm, 10401196, Schleicher & Schuell; www.s-und-s.de)
- SDS-polyacrylamide gel electrophoresis equipment
- Water bath (boiling)

1. Remove the tissue culture medium from the siRNA-treated cells cultivated in 24-well plates (from Protocol 5). Rinse the cells once with 200 µl of PBS, and add 200 µl of trypsin-EDTA. Incubate for 1 minute at 37°C; suspend the cells and add 800 µl of DMEM medium to quench the trypsin.
2. Transfer the suspended cells to a chilled 1.5-ml centrifugation tube. Collect the cells by centrifugation at 3000 rpm (700*g*) for 4 minutes at 4°C. Resuspend the cell pellet in ice-cold PBS and centrifuge again.
3. Remove the supernatant and add 25 µl of 90°C 2x concentrated Laemmli SDS sample buffer to the cell pellet obtained from one well of a 24-well plate. Incubate the sample for 3 minutes in a boiling water bath and vortex.
4. Separate the proteins by SDS-polyacrylamide gel electrophoresis using an acrylamide concentration appropriate to resolve the molecular weight of the targeted protein (Sambrook et al. 2001).

   We have separated proteins on minigels, which were run at a constant 10 mA.
5. Transfer proteins from the gel to a nitrocellulose membrane using electrotransfer buffer. Our minigels are electroblotted onto the membrane using a Bio-Rad Trans-Blot cell at 333 mA for 30 minutes in the cold room.
6. Verify the protein transfer by Ponceau S staining of the transfer membrane.
7. Incubate the membrane in blocking solution for 1 hour at 37°C.
8. Replenish the blocking solution with fresh blocking solution and add the primary antibody at the appropriate dilution. Incubate for 1–2 hours at 37°C.
9. Wash the blot four times with TBST for 10 minutes.
10. For ECL detection, incubate the blot with either HRP-conjugated rabbit anti-mouse or HRP-conjugated swine anti-rabbit antibodies at a dilution of 1:20,000 in blocking solution for 1–2 hours at 37°C.
11. Perform ECL detection according to the protocol described by the manufacturer (www.amersham.co.uk).

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